INFECTION AND IMMUNITY, Feb. 1999, p. 782–788 Vol. 67, No. 2
0019-9567/99/$04.00+0

Strategy for Cross-Protection among *Shigella flexneri* Serotypes

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Received 31 July 1998/Returned for modification 1 October 1998/Accepted 17 November 1998

Based upon the lipopolysaccharide (LPS) structure and antigenicity of *Shigella* group B, a strategy for broad cross-protection against 14 *Shigella flexneri* serotypes was designed. This strategy involves the use of two *S. flexneri* serotypes (2a and 3a), which together bear the all of the major antigenic group factors of this group. The novel attenuated strains used in these studies were *S. flexneri* 2a strain CVD 1207 (Δ*guaB-A* Δ*virG* Δ*set1* Δ*sen*) and *S. flexneri* 3a strain CVD 1211 (Δ*guaB-A* Δ*virG* Δ*sen*). Guinea pigs were immunized with an equal mixture of these strains and later challenged (Sereny test) with a wild-type *S. flexneri* serotype 1a, 1b, 2b, 4b, 5b, Y, or 6 strain of demonstrated virulence in the same model. Guinea pigs that were immunized with these two vaccine strains produced serum and mucosal antibodies that cross-reacted with all the *S. flexneri* serotypes tested (except of *S. flexneri* serotype 6) as assessed by enzyme-linked immunosorbent assay, immunoblotting, and slide agglutination. Furthermore, the combination vaccine conferred significant protection against challenge with *S. flexneri* serotypes 1b, 2b, 5b, and Y but not with serotypes 1a, 4b, or (as predicted) 6.

One hundred years after the discovery of the Shiga bacillus (later known as *Shigella dysenteriae* type 1) in Japan, shigellosis continues to be a major public health problem that kills hundreds of thousands of children in the developing world (18). The genus *Shigella* is now divided into four species or groups and at least 47 serotypes based on their biochemical and/or lipopolysaccharide (LPS) characteristics: *S. dysenteriae* (group A, 13 serotypes), *S. flexneri* (group B, 15 serotypes), *S. boydii* (group C, 18 serotypes), and *S. sonnei* (group D, 1 serotype). The World Health Organization and the Institute of Medicine (18, 42) consider the development of a vaccine against shigellosis a priority for developing countries. However, attainment of this goal has been hindered by the large number of serotypes, since it is thought that protective immunity is directed primarily against the *Shigella* O antigens and that protection is therefore serotype specific (6, 11, 13, 15, 17, 32). However, although *Shigella* spp. of any of the 47 serotypes are able to cause diarrhea and dysentery in humans, their prevalence is not evenly distributed. Of critical public health importance are *S. sonnei*, as the most prevalent *Shigella* spp. (with a unique serotype) in industrialized countries and of increasing prevalence in some Latin American countries (11); *S. dysenteriae* type 1, able to cause explosive pandemics resulting in high morbidity and mortality (1, 15, 23, 31); and *S. flexneri*, the most prevalent endemic group (comprising 15 serotypes) found in developing countries (7, 11, 20, 30). Therefore, although it may be impractical to construct a vaccine against all *Shigella* serotypes, a vaccine could be developed to protect against the most prevalent serotypes. Nonetheless, vaccine development still must address the 15 different serotypes of *S. flexneri*, which tend to be unevenly distributed in any given geographic area (7, 11, 20, 30). In this regard, while there are no significant cross-reactions among *Shigella* serotypes in groups A (*S. dysenteriae*, 13 serotypes) and C (*S. boydii*, 18 serotypes), there are major cross-reactions among 14 of the 15 serotypes included in *Shigella* group B (8). This is explained by the fact that the *S. flexneri* serotypes (with the exception of serotype 6) have some degree of antigenic relatedness attributable to a common repeating tetrasaccharide unit, α-L-Rha*p*1→2-α-L-Rha*p*1→3-α-L-Rha*p*1→3β-D-Glc*p*NAc1, to which α-D-glucopyranosyl and *O*-acetyl groups are added, providing the basis for their "type" (i.e., I to VI) and "group" (i.e., 3,4, 6, and 7,8) antigenic factors (3, 8). Rabbit antisera raised against the specific type and group antigenic factors are routinely used by clinical microbiologists in agglutination reactions to identify the *S. flexneri* serotypes (8, 12). Van De Verg et al. (38) reported that challenge or immunization with *S. flexneri* 2a (type II, group 3,4) elicited cross-reacting antibodies with *S. flexneri* serotypes that bear the group factor 3,4 or the type factor II in humans, monkeys, and guinea pigs. Lindberg et al. (22) designed a strategy of cross-protection based on an attenuated *S. flexneri* Y strain which exclusively bears the common tetrasaccharide unit serologically identified as antigenic group factor 3,4. However, the fact that significant cross-protection between most *S. flexneri* serotypes has not been found is not surprising, given the antigenic variability conferred by their type and group factors (8, 12). For example, the addition of an *O*-acetyl group on the third rhamnose or of an α-D-glucopyranosyl on the first rhamnose provides the antigenic group factors 6 and 7,8, respectively, which in many cases block the antigenicity of group factor 3,4. This is the case with *S. flexneri* serotypes 1b, 3a, 3c, and 4b (group factor 6) or serotypes 2b, 3a, 4c, 5b, and X (group factor 7,8) (Table 1). Therefore, based on the antigenic characteristics of the *S. flexneri* LPS presented above, we decided to investigate whether a mucosally administered attenuated vaccine consisting of a combination of serotypes 2a and 3a could protect against the rest of the *S. flexneri* serotypes. We selected *S. flexneri* serotypes 2a and 3a because together they bear the LPS group factors (3,4, 6, and 7,8) present in the *S. flexneri* group and two of the most prevalent type factors (II and III), providing the broadest spectrum with the minimum number of serotypes (Table 1). To test this strategy, we constructed attenuated strains of *S. flexneri* 2a and 3a based on the attenuation conferred by the previously published Δ*guaB-A* and Δ*virG* mutations (27) (Table 2). In addition, the recently described *Shigella* enterotoxin 1 (ShET1) (9, 10, 26) was ge-

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TABLE 1. Cross-reaction among *S. flexneri* serotypes

Vaccine/ O antigen	Cross-reaction with *S. flexneri* serotype:														
	1a	1b	2a	2b	3a	3b	3c	4a	4b	4c	5a	5b	6	X	Y
S. flexneri 2a CVD 1207															
Type factor II			+	+											
Group factors 3,4	+		+			+		+			+				+
S. flexneri 3a CVD 1211															
Type factor III					+	+	+								
Group factor 6		+			+	+	+		+						
Group factors 7,8				+	+					+		+		+	

netically inactivated in the *S. flexneri* 2a vaccine candidate and *Shigella* enterotoxin 2 (ShET2) (25) was inactivated in both the *S. flexneri* 2a (strain CVD 1207) and 3a (strain CVD 1211) vaccine candidates (Table 2).

MATERIALS AND METHODS

Bacterial strains and media. The strains used in this study are listed in Table 3. Wild-type *Shigella* strains were grown on Trypticase soy agar (TSA) (BBL Becton Dickinson, Cockeysville, Md.) with 0.01% Congo red dye (CR) (Sigma, St. Louis, Mo.) (TSA-CR). The same medium was supplemented with guanine (Sigma) (10 mg/liter) to grow the Δ*guaB-A* strains, arsenite (6 μmol/liter) (Sigma), carbenicillin (50 μg/ml), or kanamycin (50 μg/ml) when appropriate.

Selection of virulent strains. To be tested for virulence, the wild-type strains (Table 3) were initially screened by CR uptake (24) and PCR amplification of *virG* by using a method and primers that were previously described (27, 29). A gentamicin protection assay in HeLa cells was performed with positive strains as described previously (27, 29), and intracellular organisms were recovered after 4 h of culture. HeLa cell-invasive organisms were passaged in guinea pig conjunctiva and recovered from a purulent keratoconjunctivitis (Sereny test) (34). In a second guinea pig passage, an infectivity dose of 10^8 CFU was tested and Sereny-positive organisms were isolated and stored at −86°C as virulent stock cultures.

Inactivation of ShET1 in strain CVD 1205, yielding *S. flexneri* 2a strain CVD 1206. *S. flexneri* 2a strain CVD 1205 (Δ*guaB-A* Δ*virG*) (27) was derived from the wild-type *S. flexneri* 2a strain 2457T, which is known to be virulent based on experimental challenge studies in guinea pigs (28, 29) and adult volunteers (21). Details of the construction, characterization, and guinea pigs immunogenicity of strain CVD 1205 were published recently (27). The *set1* operon is present in all *Shigella flexneri* serotype 2a strains but is rare in isolates of other *Shigella* serotypes (26). The construction of a Δ*set1* allele, with deletion of 85% of the subunit A of *set1*, and the consequent inactivation of the ShET1 enterotoxic activity were recently demonstrated by using Ussing chambers and in vivo perfusion experiments in rabbits (9). A suicide deletion cassette was constructed by cloning the Δ*set1* allele in pFM307 (27), yielding pFM804B. This deletion cassette was used to exchange the Δ*set1* allele for the proficient *set1* allele in CVD 1205 by previously described methods (27). Clones in which *set1* was successfully deleted were selected by the lack of DNA hybridization with a 52-bp probe (5′-CCTGGCCGGGCGGGCAAAACAACCCGTTATCTTTCATGGTCAGCTGACCGG-3′) representing a deleted portion of the *set1A* gene. The deletion mutation in an arbitrarily selected clone was confirmed by PCR amplification of the truncated allele with the primers 5′-CGGGATCCCGGCCACCGGTTATGGCACCAATGAATACTGCGTTAT-3′ and 5′-GCTCTAGAGCCTGGGCCCCCTGAACTGGACATACGACAAAACATC-3′ and a protocol consisting of 94°C for 40 s, 60°C for 40 s, and 70°C for 4 min, for 30 cycles.

Inactivation of ShET2 in strain CVD 1206 and construction of *S. flexneri* 2a strain CVD 1207. ShET2 is a 62.8-kDa single-moiety protein encoded by the invasion plasmids of practically all *Shigella* serotypes (25). The Δ*sen* allele was produced by PCR amplification and fusion of two 700-bp DNA segments that include the N and C termini of *sen* minus 300 bp corresponding to the putative active site in the N-terminal region of *sen*, as was done in previously described methods (27). The resulting Δ*sen* allele was cloned into the suicide vector pFM307 (27). In addition, the proficient *sen* of *S. flexneri* 2a was cloned in pBluescript to serve as a positive control in corroborating the inactivation of ShET2 in Δ*sen*. Supernatants from *Escherichia coli* DH5α (pBluescript::*sen*) were assessed in Ussing chambers as previously described (9, 10, 25).

In addition, the *ars* operon, conferring resistance to arsenite, was cloned in the Δ*sen* locus. The 5-kbp arsenic resistance operon of R factor R773 was obtained as a *Hin*dIII fragment from pUM1 (4) (kindly provided by B. P. Rosen, Wayne State University, Detroit, Mich.) and cloned under the regulation of p_{tac} in pKK223-3 (Pharmacia, Piscataway, N.J.). A p_{tac}-*ars* *Nae*I-*Dra*I blunt-ended segment was cloned in the middle of the Δ*sen* allele in pFM307::Δ*sen*, yielding pFM220B. The Δ*sen*::p_{tac}-*ars* allele was exchanged for the proficient *sen* gene in *S. flexneri* 2a strain CVD 1206 (Δ*guaB-A* Δ*virG* Δ*set1*) by previously described methods (27), except that arsenite was added to the medium throughout the procedure. The *S. flexneri* 2a invasion plasmid containing the Δ*virG* and Δ*sen*::p_{tac}-*ars* mutations was named pFN110. In addition, supernatants of strain CVD 1207 were tested in Ussing chambers mounted with rabbit small intestinal mucosa as previously described (9, 10, 25).

Strain CVD 1207 as a donor of a virulence plasmid containing Δ*virG*, Δ*sen*, and the arsenite resistance marker. The *S. flexneri* 3a wild-type virulent strain J17B was originally isolated by S. Formal in Tokyo, Japan, in the 1970s. This strain agglutinates with group B, type factor III, group factors 6 and 7,8 antisera but not with group factor 3,4 antiserum (Table 1), and it is susceptible to all the commonly used antibiotics that were tested (data not shown). The invasion plasmid pFN110 was transferred from strain CVD 1207 to strain J17B by slight modifications of previously published methods described by Sansonetti et al. (33). Briefly, (i) *S. flexneri* 2a strain CVD 1207 (Δ*guaB-A* Δ*virG* Δ*set1* Δ*sen*) was electroporated with pF′$_{ts}$114*lac*::Tn*5*, and CVD 1207(pF′$_{ts}$114*lac*::Tn*5*) clones were selected with kanamycin (33); (ii) a suitable recipient was prepared by selecting a strain J17B clone that had spontaneously lost its invasion plasmid (as evidenced by lack of CR dye uptake and lack of hybridization with a *virG* probe [27, 29]) (J17Bavir) and electroporating it with pBluescript (Stratagene, La Jolla, Calif.) to give a temporary selection marker (ampicillin-carbenicillin); (iii) late-log-phase broth cultures of strain CVD 1207(pF′$_{ts}$114*lac*::Tn*5*) and J17Bavir (pBluescript) were mated on TSA medium for 4 h; (iv) clones of J17Bavir (pBluescript) that had acquired the *ars*-tagged virulence plasmid pFN110 [J17B (pFN110, pBluescript)] were selected on TSA medium containing arsenite and carbenicillin; (v) selected J17B(pFN110, pBluescript) clones were plated in replicate on TSA medium containing carbenicillin and arsenite and TSA medium supplemented with kanamycin to select for clones that were not transfected with pF′$_{ts}$114*lac*::Tn*5*; and (vi) after an overnight incubation at room temperature on Trypticase soy broth supplemented with guanine and 6 μM arsenite, carbenicillin-sensitive clones (that had spontaneously lost pBluescript) were selected by replica plating on medium containing arsenite or arsenite and carbenicillin. In addition, the presence of pFN110 in J17B(pFN110) was confirmed by PCR

TABLE 2. Attenuating deletion mutations in *S. flexneri* 2a strain CVD 1207 and *S. flexneri* 3a strain CVD 1211

Vaccine strain(s)	Mutation	Phenotype
S. flexneri 2a and 3a (CVD 1207 and CVD 1211)	Δ*guaB-A*	Inactivates de novo synthesis of GMP, GDP, and GTP Decreased invasion rate (27) Decreased intracellular growth (27)
	Δ*virG* (Δ*icsA*)	Inactivates VirG No intracellular and intercellular spreading (2)
	Δ*sen*::P_{tac}-*ars*	Inactivates enterotoxin ShET2 (25) High-level resistance to arsenite Allows the identification of vaccine strains Permits transfer of the invasion plasmid in the construction of new vaccine strains
S. flexneri 2a (CVD 1207)	Δ*set1A*	Inactivates enterotoxin ShET1 (9, 10, 26)

TABLE 3. Wild-type *S. flexneri* strains used in this study

S. flexneri serotype	Strain	Collection[a]
1a	3055-89	CDC
1b	3155-96	CDC
2a	2457T	CVD
2b	3346-87	CDC
3a	J17B	CVD
4b	3143-94	CDC
5b	5107-82	CDC
Y	3152-92	CDC
6	CCHO-60	CVD

[a] CDC, Centers for Disease Control and Prevention, Atlanta, Ga.; CVD, Center for Vaccine Development, Baltimore, Md.

amplification of the Δ*sen*::*ars* allele with the TaqPlus long PCR system (Stratagene, La Jolla, Calif.), primers 5′-GCTCTAGAGCAGATAATATTCAGCTTTTTATATTCTTCATAATTTCCAGA-3′ and 5′-GCTCTAGAGCACCTAGGATGGTAAGTACAGAAAACTTCAAAAAAGTTAAG-3′, and the cycling conditions 94°C for 30 s, 50°C for 30 s, and 68°C for 8 min, for 25 cycles.

The deletion mutation in the *guaB-A* operon in J17B(pFN110) was created by homologous recombination with the Δ*guaB-A* allele in pFM726A as described for the construction of strain CVD 1205 (27).

Safety (Sereny) test. The guinea pig purulent keratoconjunctivitis test was used with slight modifications of the method described by Sereny (34). Briefly, 12 Hartley guinea pigs (3 animals per group) were randomized to be inoculated in their conjunctival sac with 10 μl of a suspension containing 10^9 CFU of the *S. flexneri* wild-type strain 2457T or J17B or the vaccine strain CVD 1207 or CVD 1211. Follow-up, grading of inflammation, and statistical analysis were performed as described previously (27).

Immunizations and sample collections. Bacterial strains were cultured overnight at 37°C on TSA-CR supplemented with guanine, harvested on phosphate-buffered saline (PBS), and brought to the desired concentration (as measured by determining the optical density at 600 nm). In a preliminary study, *S. flexneri* 2a CVD 1207 and *S. flexneri* 3a CVD 1211 were individually evaluated as described previously (27). For each cross-protection study, 15 Hartley guinea pigs (weighing ≥300 g) were immunized intranasally (27, 28) with 10^{10} CFU each of CVD 1207 and CVD 1211, suspended in 100 μl of PBS; 10 guinea pigs received 2×10^{10} CFU of *E. coli* HS as placebo controls. Immunizations were performed on days 0 and 14 in animals previously anesthetized subcutaneously with ketamine HCl (40 mg/kg) (Fort Dodge Laboratories, Fort Dodge, Iowa) and xylazine (5 mg/kg) (Bayer, Shawnee Mission, Kans.). Tears were collected on days 0, 14, and 30 to 35 postimmunization as described previously (27, 28); sera were obtained on days 0 and 30 to 34 by anterior vena cava puncture (41) under intraperitoneal anesthesia with ketamine HCl and acepromazine maleate (1.2 mg/kg) (Ayerst Laboratories, Inc., New York, N.Y.).

LPS extraction. Cultures of every wild-type serotype tested were obtained from frozen stock and grown overnight on TSA-CR, and their type and group factors were confirmed by agglutination with the corresponding specific antisera (12). Bacteria were suspended in Luria-Bertani broth and incubated at 37°C with shaking overnight. *S. flexneri* LPS from each wild-type strain (Table 3) was prepared by the method of Westphal and Jann (40) and further purified by the procedures of Thomashow and Rittenberg (36). Briefly, LPS was extracted from whole cells with hot phenol and the aqueous phase was collected, dialyzed, and treated successively for 1 h each with RNase A (100 μg/ml), DNase I (50 μg/ml plus 1 mM $MgCl_2$), and pronase (250 μg/ml). EDTA (5 mM) was added, and the phenol extraction was repeated. After dialysis, the aqueous material was centrifuged at 107,000 × *g* for 2 h and the sedimented LPS was suspended in water and lyophilized. Stock solutions of LPS of each serotype were prepared in gradient-pure water at 2 mg/ml prior to use.

ELISA. Immunoglobulin A (IgA) antibodies to each specific *S. flexneri* LPS were determined by enzyme-linked immunosorbent assay (ELISA) with rabbit anti-guinea pig IgA antibody (Bethyl Lab., Inc., Montgomery, Tex.) followed by phosphatase-conjugated goat anti-rabbit IgG antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). IgG antibodies were determined by ELISA with a phosphatase-conjugated goat anti-guinea pig IgG (Bethyl Lab., Inc.) as described previously (28, 29). ELISA titers were log transformed and compared by Student's *t* test as described previously (27–29).

Immunoblots. Stock LPS solutions were mixed with equal volumes of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample lysis buffer and boiled for 10 min. Samples (30 μl containing 30 g) of each LPS preparation were electrophoresed in replicate sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (15% polyacrylamide). One gel was oxidized with periodic acid as described by Tsai and Frasch (37) and silver stained (Bio-Rad, Hercules, Calif.) to confirm the presence of LPS ladders (data not shown); LPS from other gels were transferred to nitrocellulose. Tears and sera were pooled from 60 immunized animals from four different experiments and used at a concentration of 1:100 for Western immunoblotting. The presence of specific anti-LPS IgA in tears and IgG in serum was demonstrated by using the same commercial secondary antibodies as above, and the blots were developed with the phosphate substrate chromagen (Western Blue; Promega, Madison, Wis.).

Agglutination cross-reactions. Cultures of each wild-type serotype tested were grown at 37°C overnight on TSA-CR. Pools of sera from immunized animals were used neat or in 1:2 to 1:32 dilutions in PBS. Three or four colonies of each culture were mixed with a wooden toothpick with 15 μl of neat or diluted sera on a glass slide and rocked. The pooled serum dilution in which bacteria did not agglutinate after 2 min was considered negative.

Protective efficacy in guinea pigs. In each experiment, the guinea pigs immunized with the polyvalent vaccine or with *E. coli* HS were inoculated in one of their conjunctival sacs with 10^8 CFU of one of the wild-type *S. flexneri* strains listed in Table 3. Guinea pigs were examined daily for 5 days, and inflammatory responses were graded with a severity score as described previously (28). Briefly, 0 = normal eye indistinguishable from contralateral noninoculated eye, 1 = lacrimation or eyelid edema, 2 = 1 plus mild conjunctival hyperemia, 3 = 2 plus slight exudate, and 4 = full purulent keratoconjunctivitis. The individuals examining the guinea pigs and scoring the results were blinded as to which strain(s) (vaccine or placebo) had been used for immunizations. The overall frequency of occurrence of inflammation of any severity (severity score, 1 to 4) in the vaccine and control groups was compared by Fisher's exact test. The statistical significance in peak severity scores was calculated by a nonparametric sum of ranks (Mann-Whitney test).

RESULTS

Construction of vaccine strains. The suicide deletion cassette pFM804B was used to exchange the Δ*set1* allele for the proficient *set1* allele in CVD 1205, yielding *S. flexneri* 2a strain CVD 1206 (Δ*guaB-A* Δ*virG* Δ*set1*). The specific deletion in the *set1* operon was confirmed by the lack of DNA hybridization with a 50-bp probe representing a deleted portion of the *set1A* gene and by the PCR amplification of Δ*set1* (Fig. 1). The deletion mutation of the gene encoding ShET1 (*set1*) and ShET2 (*sen*) was performed in *S. flexneri* 2a strain CVD 1205 (Δ*guaB-A* Δ*virG*) (27), yielding *S. flexneri* 2a strain CVD 1207 (Δ*guaB-A* Δ*virG* Δ*set1* Δ*sen*). The lack of enterotoxic activity of strain CVD 1207 was confirmed by experiments in Ussing chambers (data not shown). The Δ*virG* Δ*sen Shigella* invasion plasmid pFN110 of CVD 1207 (pF′$_{ts}$114*lac*::Tn*5*) was inserted into *S. flexneri* 3a J17B by conjugation. Those J17B (pFN110) clones that were also transfected with pF′$_{ts}$114*lac*::Tn*5* (50% occurrence) were identified by their resistance to kanamycin. Only J17B(pFN110) clones susceptible to kanamycin were selected. Figure 2 shows the PCR amplification of the

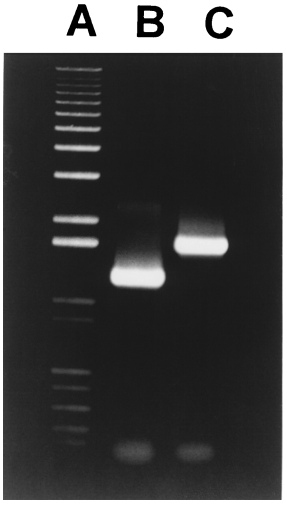

FIG. 1. Agarose gel (1% agarose) stained with ethidium bromide, showing 1-kbp ladder molecular weight markers (lane A), PCR amplification of Δ*set1* from *S. flexneri* 2a strain CVD 1206 (1.3 kbp) (lane B), and PCR amplification of wild-type *set1* from *S. flexneri* 2a strain CVD 1205 (1.6 kbp) (lane C).

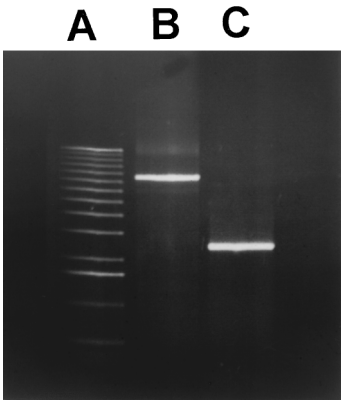

FIG. 2. PCR amplification of Δ*sen*::P_{tac}-*ars* from *S. flexneri* 3a strain CVD 1211 after conjugation with *S. flexneri* 2a strain CVD 1207 (pF′$_{ts}$114*lac*::Tn*5*). Lanes: A, 1-kbp ladder molecular weight markers; B, Δ*sen*::P_{tac}-*ars* (7.5 kbp) from strain CVD 1211; C, *sen* (2.2 kbp) from wild-type *S. flexneri* 3a strain J17B.

Δ*sen*::P_{tac}-*ars* allele in the invasion plasmid of J17B(pFN110) after conjugation. Consequently, the *guaB-A* deletion mutation was performed in strain J17B(pFN110), yielding *S. flexneri* 3a strain CVD 1211 (Δ*guaB-A* Δ*virG* Δ*sen*). Strains CVD 1207 and CVD 1211 do not grow in minimum medium unless it is supplemented with guanine as described previously (27). In addition, these strains are resistant to at least 6 μM arsenite in the medium but no growth was obtained at this concentration of arsenite with any other *Shigella* strain (belonging to the four *Shigella* groups) tested.

Sereny studies. No inflammatory response was observed in the conjunctivas of three guinea pigs that received 10^9 CFU of *S. flexneri* 2a strain CVD 1207 or three guinea pigs that received 10^9 CFU of *S. flexneri* 3a strain CVD 1211. Full purulent keratoconjunctivitis was observed in all controls that received the virulent strains 2457T and J17B.

Assessment of cross-reactivity elicited by an *S. flexneri* 2a/3a vaccine against the LPS of other *S. flexneri* serotypes. As determined by ELISA (Table 4), two doses of the polyvalent *S. flexneri* 2a/3a vaccine (CVD 1207/CVD 1211) elicited IgG antibodies in serum that strongly cross-reacted with *S. flexneri* LPS of serotypes 1a and 1b (Table 4). In contrast, the IgA antibodies elicited in tears by this combination vaccine showed an extensive cross-reaction that included *S. flexneri* serotypes 1a, 1b, 2b, 4b, 5b, and Y (Table 4). Interestingly, the cross-reaction against *S. flexneri* 2b by ELISA was modest even though this particular serotype shares antigenic type factor II with *S. flexneri* 2a and group factor 7,8 with *S. flexneri* 3a.

The strategy of cross-protection presented herein is based on the pattern of cross-reactivity given by the antigenic group factors, which forms the basis for the identification of the *S. flexneri* serotypes in agglutination reactions. Therefore, we considered it relevant to analyze the serum agglutination obtained by using a vaccine that includes all antigenic group factors. As shown in Table 4, pooled sera from immunized guinea pigs agglutinated heterologous *S. flexneri* serotypes, in most cases at a dilution of 1:4. The degree of cross-agglutination seemed independent of the geometric mean titer of IgG in serum, obtained by ELISA for that particular serotype. As expected, no cross-agglutination was observed with *E. coli* HS or *S. flexneri* serotype 6.

Furthermore, IgG and IgA immunoblotting performed with pools of sera and tears from immunized animals demonstrated a distinct pattern of cross-reactivity. As shown in Fig. 3, immunization with the *S. flexneri* 2a/3a vaccine elicited IgG in serum and IgA in tears that strongly reacted with the homologous *S. flexneri* 2a and *S. flexneri* 3a LPS. A more modest cross-reaction was observed with the LPS ladder of *S. flexneri* 1b (type I; group 6) and 2b (type II; group 7,8). However, a strong cross-reaction was observed against the low-molecular-weight LPS ladder of all *S. flexneri* serotypes tested except serotype 6. Noticeably, the mucosal IgA LPS reactions against the homologous and heterologous *S. flexneri* serotypes (Fig. 3B) were more evident than the serum IgG ones (Fig. 3A). No cross-reaction was observed against the LPS ladder of serotype 6 in the IgG or the IgA immunoblots (Fig. 3).

Protection elicited by *S. flexneri* 2a strain CVD 1207 and *S. flexneri* 3a strain CVD 1211 against challenge with their homologous serotypes. In preliminary experiments, we tested the ability of the individual vaccine strains to protect against their homologous serotypes. *S. flexneri* 2a strain CVD 1207 conferred 85% protective efficacy to guinea pigs against keratoconjunctivitis produced by wild-type strain 2457T ($P = 0.05$). Likewise, *S. flexneri* 3a strain CVD 1211 conferred 75% protection against conjunctival challenge with wild-type strain J17B ($P = 0.01$).

Cross-protection among *S. flexneri* serotypes elicited by the *S. flexneri* 2a/3a vaccine. The protection conferred by the combination of *S. flexneri* 2a and *S. flexneri* 3a vaccine strains against other *S. flexneri* serotypes is shown in Table 5. There was considerable variation in the degree of cross-protection conferred by this vaccine against the heterologous serotypes. The highest cross-protection (92.5%) was observed against *S. flexneri* 2b, which, as discussed above, shares with the com-

TABLE 4. Cross-reactivity of IgG in serum and IgA in tears and cross-agglutination of immune sera to various *S. flexneri* serotypes after immunization with an *S. flexneri* 2a/3a (CVD 1207/CVD 1211) combination vaccine

Immunization	LPS tested	Cross-reacting O antigens	LPS cross-reactivity		
			IgG GMT[a]	IgA GMT	Agglutination[b]
Vaccine	2a	II; 3,4	536	1,902	1:16
Placebo	2a	None	15	46	−
Vaccine	3a	III; 6,7,8	220	8,458	1:16
Placebo	3a	None	15	20	−
Vaccine	1a	3,4	579	702	1:2
Placebo	1a	None	12.5	40	−
Vaccine	Y	3,4	64	1,640	1:4
Placebo	Y	None	12.5	20	−
Vaccine	1b	6	520	1,724	1:4
Placebo	1b	None	25	26	−
Vaccine	4b	6	44	1,769	1:4
Placebo	4b	None	15	20	−
Vaccine	2b	II; 7,8	50	884	1:4
Placebo	2b	None	27	20	−
Vaccine	5b	7,8	29	1,345	Neat
Placebo	5b	None	12.5	20	−
Vaccine	6	None	15	145	−
Placebo	6	None	12.5	20	−

[a] GMT, geometric mean titer.
[b] Pool of serum dilution that produced slide agglutination. −, Negative slide agglutination with neat antiserum.

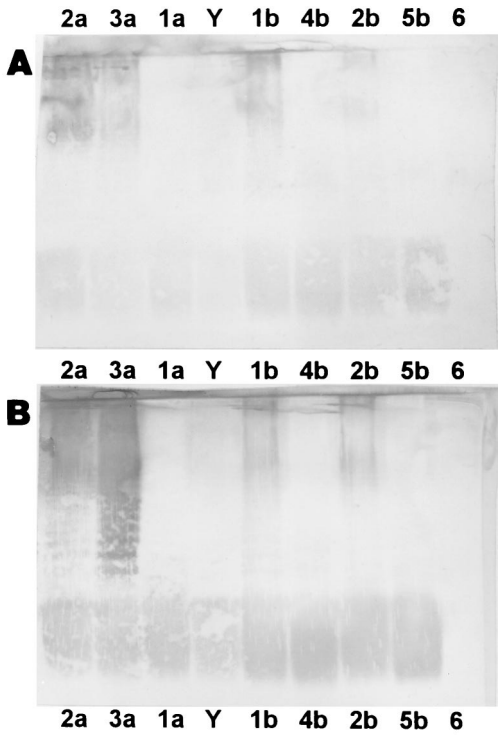

FIG. 3. Cross-reactions against *S. flexneri* LPS after immunization with the attenuated *S. flexneri* 2a/3a (CVD 1207/CVD 1211) vaccine. (A) Immunoblotting with pooled guinea pig sera showing IgG cross-reactions. (B) Immunoblotting with pooled guinea pig tears showing IgA cross-reactions. The designation of each *S. flexneri* serotype is indicated above and below each lane.

bination vaccine its type and group factors (Table 5). Interestingly, this high degree of protection was achieved despite a comparatively low cross-reacting serum IgG response against its LPS (Table 4). At the other extreme was the very low protection (protective efficacy, 20% [not significant]) achieved against *S. flexneri* type 4b strain 3143-94. However, despite the low "total" protection obtained against this serotype, the severity of the inflammatory response that was blindly recorded in vaccinees was significantly milder than that in the placebo controls (Table 5). In contrast, the attack rate and the degree of inflammation in guinea pigs that received the combination vaccine or the placebo control and were challenged with *S. flexneri* serotype 6 were basically equivalent.

DISCUSSION

There are major cross-reactions among the multiple serotypes included in *Shigella* group B (*S. flexneri*) (8), and data from experimental observations (35, 39) and clinical trials (5) support the belief that cross-protection may be conferred by some of the members of this group against other serotypes of the same group. Weil and Farsetta (39) reported 50 years ago that mice immunized with *S. flexneri* III-Z (later known as *S. flexneri* 3a [type III; group 6, 7,8]) conferred cross-protection against challenge with wild-type *S. flexneri* I to III (later known as *S. flexneri* 1b [type I; group 6]) but much less protection against challenge with *S. flexneri* II-W and VI-Boyd 88 (later known as *S. flexneri* 2a [type II; group 3,4] and *S. flexneri* 6, respectively). Twenty-five years later, Sereny et al. (35) reported that immunization of guinea pigs with *S. flexneri* 4b (type IV; group 6) protected them against challenge with the homologous wild-type *S. flexneri* 4b and the heterologous *S. flexneri* 3a (type III; group 6, 7,8). Although the association was not made at the time, in these two instances the strains used to immunize the animals and the heterologous strains to which they elicited protection possessed the antigenic group factor 6. Other reports are more difficult to analyze because of the lack of identification of the specific subserotypes involved in the studies. An example is the study by Cooper et al. (5), in which serum from children immunized with *S. flexneri* types II, III, and VII (later known as *S. flexneri* serotypes 2, 3, and X,

TABLE 5. Cross-protection after immunization with an *S. flexneri* 2a/3a (CVD 1207/CVD 1211) combination vaccine[a]

Immunization	Challenge		Attack rate[b]	Efficacy (%)	P^c	Severity score[d]	P^e
	Serotype	Strain					
Vaccine	1a	3055-89	8/15 (53)	42	0.065	2	0.06
Placebo	1a	3055-89	8/9 (89)			3.4	
Vaccine	Y	3152-92	3/12 (25)	80	<0.001	0.5	<0.001
Placebo	Y	3152-92	9/10 (90)			3.6	
Vaccine	1b	3155-96	6/15 (40)	56	0.016	1.3	0.006
Placebo	1b	3155-96	9/10 (90)			3.1	
Vaccine	4b	3143-94	12/15 (80)	20	0.224	2.7	0.015
Placebo	4b	3143-94	9/9 (100)			4	
Vaccine	2b	3346-87	1/15 (7)	92	<0.001	0.2	<0.001
Placebo	2b	3346-87	8/9 (89)			3.4	
Vaccine	5b	5107-82	4/14 (29)	64	0.018	0.9	0.02
Placebo	5b	5107-82	8/10 (80)			2.3	
Vaccine	6	CCHO-60	10/15 (67)	4	0.607	2.5	0.44
Placebo	6	CCHO-60	7/10 (70)			2.4	

[a] *S. flexneri* 2a strain CVD 1207 conferred 85% protection against wild-type *S. flexneri* 2a strain 2457T; *S. flexneri* 3a strain CVD 1211 conferred 75% protection against wild-type *S. flexneri* 3a strain J17B.
[b] Number positive in Sereny test/total number tested (percent positive).
[c] Fisher exact test on attack rate, vaccine versus placebo.
[d] Mean of severity score.
[e] Mann-Whitney test on severity score, vaccine versus placebo.

respectively) protected mice against the homologous serotypes and against the heterologous *S. flexneri* I (later known as *S. flexneri* serotype 1), but not against serotype VI (later known as *S. flexneri* serotype 6). As mentioned above, *S. flexneri* 6 has a different LPS structure from the rest of the group B serotypes. More recently, Lindberg et al. (22) constructed a vaccine candidate (strain SFL114) based on an attenuated strain of *S. flexneri* Y (group factor 3,4) which protected monkeys against challenge with wild-type strains of the serotypes *S. flexneri* Y (homologous), *S. flexneri* 1b (type I; group 6), and *S. flexneri* 2a (type II; group 3,4) (19). Hartman et al. (16) confirmed the capacity of the same *S. flexneri* Y vaccine to elicit protection in guinea pigs against the wild-type homologous serotype. However, immunized animals were not protected against challenge with wild-type *S. flexneri* 2a. We do not have a plausible explanation for the discrepancy observed with the two animal models. It is known that monkeys often acquire natural infections with *Shigella* spp. (14) and subsequently may be more prone than guinea pigs to produce cross-reactive immune responses (14, 38).

Reported herein is the construction of two novel vaccine candidates with a combination of deletion mutations in metabolic (*guaA* and *guaB*) and virulence (*virG*, *sen*, and *set1*) genes that give striking attenuating characteristics (Table 2). However, despite their marked attenuation, both strains were demonstrated to be immunogenic and protective in our guinea pig animal model. In addition, during construction of these strains, we inserted a nonantibiotic selection marker in the middle of the Δ*sen* allele to allow facile transfer of the virulence plasmid containing the deletion mutation in *virG* and *sen* to other vaccine strains (i.e., *S. flexneri* serotype 6, *S. dysenteriae* serotype 1) and the identification of the vaccine strain in the field. The ability to readily transfer the arsenite resistance-tagged Δ*virG* Δ*sen* virulence plasmid in *S. flexneri* 2a strain CVD 1207 (pF′$_{ts}$114*lac*::Tn*5*) was demonstrated in the construction of *S. flexneri* 3a strain CVD 1211. Sansonetti et al. (33) had previously used the F′ factor encoded in pF′$_{ts}$114*lac*::Tn*5* to transfer an invasion plasmid (pWR110) from a virulent *Shigella* strain into a plasmidless avirulent one. In this report, we have demonstrated that the same technique can be applied to transfer an invasion plasmid with specific attenuating mutations to facilitate the construction of *Shigella* vaccines.

The attenuated *S. flexneri* 2a strain CVD 1207 and *S. flexneri* 3a CVD 1211 were constructed because, in addition to belonging to serotypes that are very prevalent in developing countries, together they bear the immunodominant antigenic group factors of the *S. flexneri* group. The results presented herein demonstrate that broad cross-protection, albeit not complete, is achieved by a vaccine consisting of a combination of these two serotypes. As may be expected, given the antigenic diversity of the *S. flexneri* serotypes, the degree of cross-protection varied. Thus, a high degree of cross-protection may be achieved if the antigenic type factor as well as the group factors are covered by the combination vaccine. This was observed when the CVD 1207-plus-CVD 1211-immunized guinea pigs were challenged with a wild-type virulent strain of the *S. flexneri* 2b (type II; group 7,8) or Y (group 3,4) serotypes (Table 5). Likewise, a similar outcome may occur against virulent strains of *S. flexneri* serotypes 3b (type III; group 3,4, 6) and 3c (type III; group 6). However, we cannot rule out the possibility that a low cross-protection rate will be observed against certain serotypes, such as *S. flexneri* 4b (type IV; group 6) (20% vaccine efficacy in this study). Nevertheless, even in those cases, the vaccine may confer a significant degree of protection against the severity of disease (Table 5).

The vaccination strategy against the *S. flexneri* group presented in this paper may simplify the construction of a broad-spectrum vaccine against shigellosis. Ideally, it will be desirable for this vaccine to be flexible so that it can meet the specific needs of the geographic area to be targeted. For example, one may envision an attenuated vaccine for oral administration containing *S. flexneri* serotypes 2a and 3a (covering most of the *S. flexneri* group) and 6 (which does not cross-react with the other *S. flexneri* serotypes), to which can be added *S. sonnei* and/or *S. dysenteriae* type 1 depending on the geographic area for which these are intended. Alternatively, another *Shigella* serotype(s) may be added if it is thought to be prevalent and not covered by this vaccine (i.e., an *S. dysenteriae* 2-13 or *S. boydii* 1-18 serotype, which are usually of low prevalence) or that inadequate cross-protection is achieved against it (i.e., *S. flexneri* 4b). Thus, hypothetically, attenuated strains of five or six serotypes contained in an oral vaccine formulation could protect against the great majority of the causes of shigellosis in the world.

ACKNOWLEDGMENT

This work was supported by grant 1RO1 AI40261-01 from the National Institute of Allergy and Infectious Diseases.

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Editor: D. L. Burns